IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Gyula Hadlaczky, et. al	Art Unit	: 1638
Serial No.	: 09/724,726	Examiner	: Georgia Helmer
Filed	: November 28, 2000	Cust No.	: 20985
Conf. No.	: 7776		
Title	: ARTIFICIAL CHROMOSOMES, USES THEREOF AND METHODS FOR PREPARING ARTIFICIAL CHROMOSOMES		

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Dear Sir:

I, Steven F. Fabijanski, declare as follows:

1) I am familiar with the subject matter of the above-captioned application, which was filed on November 28, 2000.

2) I received a Bachelor's degree in Biology from the University of Miami (Florida) in 1977. I received a Ph.D. degree in Cellular and Molecular Biology from the University of Southern California in 1981. I have held post-doctoral positions at the University of Ottawa in Ottawa, Ontario, Canada and the University of Southern California in Los Angeles, California from 1982 to 1985. From 1986 to 1991, I held the position of Research Director at Paladin Hybrids, Inc.

3) I have over 20 years of experience in the areas of plant molecular biology, plant gene expression, plant tissue and cell culture and development of techniques to produce genetically modified plants and plant artificial chromosomes. I have authored or co-authored over 20 publications and I am an inventor of 15 U.S. and foreign patents.

4) I am currently Research Director at Agrisoma Biosciences Inc., located in Saskatoon, Saskatchewan, Canada. I have held this position since 2001. I also am President of FAAR Biotechnology Group Inc., located at Suite 323, 5929L Jeanne D'arc Boulevard, Orleans, Ontario, Canada K1C 7K2. I have held this position since 1992.

5) Chromos Molecular Systems, Inc., located at 8081 Lougheed Highway, Burnaby, B.C., Canada V5A 1W9, an assignee of the above-captioned application, is an owner of Agrisoma Biosciences, Inc.

6) In my capacity as researcher, myself, persons under my direction and other research groups: the Scottish Crop Research Institute in Scotland; the Danforth Plant Science Center in St. Louis, Missouri; the Hungarian Biological Research Center in Hungary; and Applicant's research group at the Plant Biotechnology Institute in Saskatoon, Saskatchewan, Canada; have studied the generation of plant artificial chromosomes and the introduction of satellite artificial chromosomes into plant cells and transgenic plants.

7) Using methods and materials described in the above-referenced application and standard methods as described herein, myself and other scientists involved in these projects have demonstrated that plant cells and whole transgenic plants can be produced that contain satellite artificial chromosomes.

As exemplified by the results shown below, we have demonstrated element-for-element and step-for-step that, by following the teachings in the application, transgenic plants containing satellite artificial chromosomes can be generated by i) introducing a DNA fragment with a selectable marker into a plant cell; ii) growing the cell under selective conditions to produce a plant cell that has incorporated the DNA into its genomic DNA, such that a plant cell containing a satellite artificial chromosome is produced; and iii) growing the resulting plant cell containing a satellite artificial chromosome under conditions that regenerate a transgenic plant, such that a transgenic plant containing a satellite artificial chromosome is produced. Further, we have demonstrated element-for-element and step-for-step that, by following the teachings in the application, that whole plants that contain satellite artificial chromosomes can be produced by i) introducing a satellite artificial chromosome into a plant cell; and ii) growing the plant cell under conditions that regenerate a transgenic plant, such that a transgenic plant containing a satellite artificial chromosome is produced.

MATERIALS AND METHODS

A. Generation of Whole Plants Containing a Satellite Artificial Chromosome

1. Construction of heterologous DNAs

Vector pAgIIa, containing two selectable markers and a sequence with homology to the pericentric DNA, was constructed using standard techniques of molecular biology. A hygromycin phosphotransferase (HPT) gene under the control of the 35S promoter (see, for example, Blochinger *et al. Mol. Cell. Biol. 4*:2929-2931) was incorporated into the vector for selection. A 334 base pair sequence with homology to tobacco pericentric sequences (Genbank Accession No. Y08422, submitted 1996; see also Genbank Accession Nos. X76056 and D76443 submitted 1993 and 1995, respectively) was constructed, containing the central AT-rich region of a tobacco rDNA intergenic spacer capable of amplification (Borisjuk *et al.* (1997) *Plant Mol. Biol. 35*:655-660). Vector pAGIIa also contains a visible marker, constructed by placing a β-glucuronidase (GUS) gene under the control of the nos promoter (Novel *et al.* (1973) *Mol. Gen. Genet. 120*:319-335; Jefferson *et al.* (1986) *Proc. Natl. Acad. Sci. USA 83*:8447-8451; US Patent No. 5,268,463; commercially available from Clontech Laboratories, Palo Alto, CA) and a detection marker containing a 234 base pair mouse major satellite DNA sequence derived from pSAT-1 (Wong *et al.* (1988) *Nucleic Acid Research, 16(24)*:11645-11661. The vector also contains a second selectable marker constructed from a phosphinothricin acetyl transferase (PAT) gene under the control of the 35S promoter, (see for example, White *et al.* (1990) *Nuc. Acids Res. 18*:1062; Spencer *et al.* (1990) *Theor. Appl. Genet. 79*:625-631; Vickers *et al.* (1996) *Plant Mol. Biol. Reporter 14*:363-368; and Thompson *et al.* (1987) *EMBO J. 6*:2519-2523).

A targeting DNA construct was constructed with homology to pericentric DNA sequences. The targeting DNA construct contains a 1.7 Kb portion of the 26S rDNA coding region (Genbank accession X52320). The targeting DNA construct was cloned into the vector pBluescript (Stratagene, La Jolla, CA).

2. Introduction of DNAs into plant cells and selection

Vector DNA and targeting DNA were introduced into tobacco cells using PEG mediated transfection. Briefly, tobacco protoplasts were isolated from established sterile tobacco plant cultures by immersion of sterile tissue in enzyme solution containing 1.2% Cellulase 'Onozuka' R-10 and 0.4% Macerozyme R-10. The protoplasts were purified by pouring through a 100 μm nylon mesh sieve, overlaid with washing solution and centrifuged at 80 x g for 10 min. Protoplasts were then resuspended at a density of 1 $x10^6$ protoplasts/ml and stored at 4°C for 1 to 2 hours prior to DNA uptake.

The vector and targeting DNAs were sterilized with chloroform and 70% ethanol before use. A protoplast suspension was mixed with vector and targeting DNA at a ratio of 1:10, followed immediately by slow addition of a polyethylene glycol (PEG) solution. As controls, salmon sperm or calf thymus DNA was added instead of the targeting DNA. The mixture was incubated at 22°C for 10-15 min, with gentle shaking. The protoplasts were resuspended and cultured at 22°C in the dark. When microcalli developed, the protoplasts were embedded in 0.6% agarose. Selection on protoplast cultures was carried out by adding hygromycin to the medium at a final concentration of 20 mg/l, 14 to 21 days after transfection.

Calli that grew on selection were cultured under selective conditions for a period of 3 - 6 months, with frequent subculturing. Standard molecular biology techniques were used to verify the presence of the vector DNA.

3. Identification of amplified DNA Molecules

GUS-expressing calli produced using vector DNA and either the targeting DNA or control DNA were subjected to two-color Fluorescent In Situ Hybridization (FISH) using two probes. The first probe was tagged with rhodamine (red fluorescence) and recognized pericentric DNA (18S rDNA) sequences endogenous to tobacco cells. The second probe recognized the detection marker (mouse major satellite sequence) in the pAgIIa vector used for transfection and was visualized with a fluorescein isothiocyanate (FITC) tag (blue-green fluorescence).

To obtain spreads of metaphase chromosomes, cells were subjected to either a single blocking protocol (colchicine treatment), or double blocking protocol (for example, treating plant cells with 5 mg/L aphidicolin for 24 hours and then 1.54 mg/ml Propyzamide for 4 hours). The blocked cells were recovered and chromosome spreads prepared and subjected to two-color FISH. Red and blue-green fluorescence was monitored to identify amplification. In general, 8-10 chromosome spreads were screened per sample. Further fluorescent image analysis was performed in a subset of the samples to overlay the probe signals and further detail chromosome structure.

4. Regeneration of Transgenic Plants containing Satellite Artificial Chromosomes

Whole plants were regenerated according to Robert *et al.*, ((1989) *Plant Molecular Biology* 13:399-309). Whole plants were regenerated from *Nicotiana tabacum* cells containing a SATAC by culturing the cells in plant culture media with a decreased auxin concentration and in the presence of a cytokinin hormone. For shoot regeneration from *Nicotiana tabacum* calli comprising the SATAC, the callus containing the SATAC was placed on basal MS media (Sigma M5519) containing 3% sucrose, 0.8% agar, with the addition of 0.5 mg of benzyladenine (BA), pH 5.8 . Shoots appeared over a period of 2 – 4 weeks. Once shoots reached a size of approximately 1.5 cm in length, they were excised and placed on basal MS media as above, but lacking BA and containing IBA (Indole Butyric Acid) to facilitate rooting. Shoots that developed roots were transferred to soil and grown in the greenhouse.

B. Generation of Transgenic Plants Containing Satellite Artificial Chromosomes by Cell Fusion

Whole regenerated plants containing a SATAC as described above were used as source material for the transfer of the SATAC to a new plant species. Using the techniques of interspecific fusion described by Evans *et al* (1980) *Plant Physiology 48*: 25-230, protoplasts from the *N. tabacum* plants containing a SATAC were isolated and fused to protoplasts of *N. glauca*, producing hybrid plants that carried the SATAC.

For interspecific fusion, protoplasts from donor (*Nicotiana tabacum* containing a SATAC, described above) and recipient plants (*Nicotiana glauca*) were isolated by digestion with an enzyme solution. The enzyme solution contained per 100 mls of solution, 1.2 g Onozuka R10, 0.4 g Macerozyme R10, and 0.2 g Driselase enzymes dissolved in a solution containing 0.32 g basal B5 medium (Sigma B5B5 G5893), 75 mg $CaCl_2$-H_2O, 58.5 mg MES, 13.69 g sucrose, 0.1 mg NAA, 0.01 mg 2,4-D, 0.02 mg BA, pH 6.0, sterile filtered.

Protoplasts from *N. tabacum* were first treated with Iodoacetate (IOA) prior to fusion to inactivate the cytoplasm and to ensure that only *N. glauca* fusion products were obtained. To treat the donor *N. tabacum* protoplasts with IOA, a fresh solution of 10 mM IOA was prepared in W5 buffer (154 mM NaCl, 125mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 5.8). 1 X 10^6 donor protoplasts incubated in this solution for 30 minutes. The IOA treatment renders the *N. tabacum* cells inactive but preserves the integrity of the chromosomes. Hence, following fusion with *N. glauca*, only those *N. glauca* protoplasts that received a SATAC carrying the selectable marker for hygromycin resistance were viable under selection with hygromycin. Approximately 1 X 10^6 protoplasts from donor and recipient plants were mixed in the presence of a 60% PEG 4000 solution. The PEG solution (50 mls) contained 30g of PEG 4000, 17 g of sucrose, 150 mg of $CaCl_2 \cdot 2H_2O$, 10 mg of $KH_2.PO_4$, pH 6.0, sterile filtered. It was prepared according to the procedure described by Kao and Saleem (1986) *J. Plant Physiol. 122*:217-225, except that the purified solution was separated from the resin by filter-sterilization.

After the two protoplast populations were mixed in the presence of PEG, a high calcium, high pH, hypotonic solution was then added to accelerate fusion. This added solution contained per 100 ml of sterile filtered solution, 4.5 g of glucose, 294 mg of $CaCl_2 \cdot 2\ H_20$, and 66.3 mg of CAPS, pH 10.5. The fused protoplasts were washed with W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 5.8). The fused protoplasts then were plated under selection in liquid media containing 20 mg/L hygromycin to recover protoplasts that received a SATAC. After a period of 2-

4 weeks, hygromycin resistant calli appeared. Calli were transferred to solid media as described by Evans *et al.* (1980) *Plant Physiology 48*: 25-230, and allowed to expand.

For shoot regeneration from *Nicotiana* hybrid calli, calli resistant to hygromycin were placed on basal MS media (Sigma M5519) containing 3% sucrose, 0.8% agar, 0.5 mg of BA, pH 5.8, as described above. Shoots appeared over a period of 2 – 4 weeks.

RESULTS

A. Generation of Transgenic Plant Containing Satellite Artificial Chromosomes

1. Generation of Plants Cells Containing Satellite Artificial Chromosomes

Following introduction of the heterologous DNAs into tobacco cells, cells were selected on hygromycin. More than 400 calli were obtained. A portion of the calli were analyzed for expression of the GUS reporter gene. A total of 31 independent GUS-expressing calli obtained using the targeting DNA or control DNAs were selected for further analysis.

The calli were subjected to two-color FISH using the probes for endogenous pericentric (18S rDNA) sequences and for the detection marker in the vector. The endogenous pericentric (rDNA) loci on tobacco chromosomes stained red, and regions of the chromosome where the vector DNA inserted stained blue-green. Where amplification of the vector DNA had occurred, medium to high blue-green signal was observed. In 7 out of 24 analyzed calli produced with targeting DNA homologous to pericentric sequences, large scale amplification of the vector sequences was observed at the chromosome level. No such amplification was observed using salmon sperm or calf thymus DNAs, indicating that targeting DNA without known homology to pericentric DNA is less efficient for stimulating large scale amplification.

One of the 7 callus lines with a medium-high vector signal was analyzed further. This line was shown to contain a chromosome that exhibited large scale "sausage" amplification and a breakage product representing a plant artificial

chromosome (plant SATAC). The SATAC was clearly visible in chromosome spreads as a small independent chromosome entity containing both amplified vector DNA and pericentric DNA. The SATAC contained amplified vector DNA as well as heterochromatic DNA. The callus line containing the plant SATAC was stably maintained in culture for over 6 months.

2. Regeneration of Transgenic Plants

Shoots were regenerated from the *N. tabacum* calli containing a SATAC (described above). The calli yielded shoots over a period of 2 – 4 weeks. These shoots were rooted and then transferred to the greenhouse for further growth. From the transferred shoots, more than 20 whole transgenic plants were obtained. Regenerated plants that contained a SATAC were identified using fluorescent *in situ* hybridization (FISH). Multiple regenerated plants were identified that had FISH signals indicating the presence of the SATAC. These plants had been regenerated from the *N. tabacum* calli independently from one another. At least 5 plants were allowed to flower and set seed.

B. Production of Transgenic Plants Containing a Satellite Artificial Chromosome by Cell Fusion

Following cell fusion of *N. tabacum* and *N. glauca* protoplasts, more than 150 hybrid calli were recovered. A portion of these hybrid calli were induced to regenerate whole plants containing a SATAC. Using the GUS visible marker gene and the hygromycin resistance selectable marker present on the SATAC, more than 50 regenerated hybrid plants were obtained that express the marker genes, consistent with the presence of the SATAC.

CONCLUSION

The above studies demonstrate that, as described in the above-captioned application, transgenic plants that contain satellite artificial chromosomes can be produced. The results demonstrate that plant cells containing a satellite artificial chromosome can be generated by (i) introducing heterologous DNA into a plant cell;

(ii) growing cells under selective conditions to produce cells that have incorporated the DNA and where the DNA undergoes amplification, such that a satellite artificial chromosome is produced; and (iii) growing the resulting plant cell containing a satellite artificial chromosome under conditions that regenerate a transgenic plant, such that a transgenic plant containing a satellite artificial chromosome is produced. The regenerated plants containing a satellite artificial chromosome flower and set seed.

In addition, the above studies demonstrate that transgenic plants can be produced containing satellite artificial chromosomes by (i) introducing a satellite artificial chromosome into a plant cell; and (ii) growing the plant cell under conditions that regenerate a transgenic plant, such that a transgenic plant containing a satellite artificial chromosome is produced. The studies demonstrate that transgenic plants containing a satellite artificial chromosome can be produced by (i) introducing a satellite artificial chromosome into a plant cell using cell fusion between protoplasts of different plant species; and (ii) growing the plant cell under conditions to regenerate a transgenic plant. The regenerated transgenic plants contain a satellite artificial chromosome. In addition, the transgenic plants express marker genes present on the satellite artificial chromosome.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Dec. 07, 2004
Date

Steven F. Fabijanski